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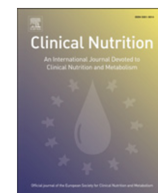

Short Communication

The effect of olive oil polyphenols on antibodies against oxidized LDL. A randomized clinical trial

Olga Castañer[a,b], Montserrat Fitó[a,*], M. Carmen López-Sabater[c], Henrik E. Poulsen[d], Kristiina Nyyssönen[e], Helmut Schröder[a], Jukka T. Salonen[f], Karina De la Torre-Carbot[c], Hans-Franz Zunft[g], Rafael De la Torre[a], Hans Bäumler[h], Antonio V. Gaddi[i], Guillermo T. Saez[j], Marta Tomás[a], Maria-Isabel Covas[a] for the EUROLIVE Study Group

[a] Cardiovascular Risk and Nutrition, Cardiovascular Epidemiology and Genetics, and Human Pharmacology and Clinical Neurosciences Research Groups, Research Institute Hospital del Mar (IMIM), Barcelona 08003, Spain
[b] University of Barcelona, Spain
[c] Department of Nutrition and Food Science, Reference Centre in Food Technology, Faculty of Pharmacy, University of Barcelona, Spain
[d] Department of Clinical Pharmacology, Rigshospitalet, University Hospital Copenhagen, Denmark
[e] Research Institute of Public Health, University of Kuopio, Finland
[f] CEO, MAS-Metabolic Analytical Services Oy, Finland
[g] German Institute of Human Nutrition, Postdam-Rehbruecke, Germany
[h] Charité-University of Medicine of Berlin, Germany
[i] GC Descovich Centre for the Study of Arteriosclerosis and Metabolic Diseases, Department of Clinical Medicine and Applied Biotechnology, Policlinico S. Orsola-Malpighi, Bologna, Italy
[j] Department of Biochemistry and Molecular Biology, faculty of Medicine CDB-HGUV. University of Valencia, Spain

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SUMMARY

Background & aim: Oxidized LDL (oxLDL) is a highly immunogenic particle that plays a key role in the development of atherosclerosis. Some data suggest a protective role of OxLDL autoantibodies (OLAB) in atherosclerosis. Our aim was to assess the effect of olive oil polyphenols on the immunogenicity of oxLDL to autoantibody generation.
Methods: In a crossover, controlled trial, 200 healthy men were randomly assigned to 3-week sequences of 25 mL/day of 3 olive oils with high (366 mg/kg), medium (164 mg/kg), and low (2.7 mg/kg) phenolic content.
Results: Plasma OLAB concentration was inversely associated with oxLDL ($p < 0.001$). Olive oil phenolic content increased OLAB generation, with the effect being stronger at higher concentrations of oxLDL ($p = 0.020$ for interaction). A direct relationship was observed between OLAB and the total olive oil phenol content in LDL ($r = 0.209$; $p = 0.014$). OLAB concentrations, adjusted for oxLDL, increased directly in a dose-dependent manner with the polyphenol content of the olive oil administered ($p = 0.023$).
Conclusion: Olive oil polyphenols promote OLAB generation. This effect is stronger at higher concentrations of lipid oxidative damage.
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1. Introduction

Olive oil is the primary source of fat in the Mediterranean diet. It contains multiple minor components such as polyphenols.[1] In the EUROLIVE study, the benefits of high-polyphenol olive oil consumption at real-life olive oil doses were directly associated with a decrease of markers of low density lipoprotein (LDL) oxidation.[2,3] Oxidation-immune mechanisms are involved in atherogenesis.[4] One of the earliest steps in the generation of oxidized LDL (oxLDL) is the peroxidation of its polyunsaturated fatty acids. The oxidative breakdown of these fatty acids forms covalent bonds with lysine

Abbreviations: oxLDL, Oxidized Low Density Lipoprotein; OLAB, OxLDL autoantibodies; OOPC, olive oil phenolic compound; PC, phenolic compound; LPC, low phenolic compound; MPC, medium phenolic compound; HPC, high phenolic compound; HPLC, high-performance liquid chromatography; GS–MS, gas chromatography coupled to mass spectrometry.

* Corresponding author. Cardiovascular Risk and Nutrition Research Group, Research Institute Hospital del Mar (IMIM-Hospital del Mar), Parc de Recerca Biomèdica de Barcelona (PRBB), Carrer Doctor Aiguader 88, Barcelona 08003, Spain. Tel.: +34 93 316 17 24; fax: +34 93 316 07 96.
E-mail address: mfito@imim.es (M. Fitó).

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residues of apolipoprotein-B which becomes immunogenic.[5] Thus, autoantibodies against oxLDL (OLAB) are generated and are detectable in serum. Some data suggest a protective role of OxLDL autoantibodies (OLAB) in atherosclerosis.[4] In this sense, OLAB concentrations decrease at high levels of lipid oxidation.[6] The aim of our study was to assess the effect of olive oil phenolic compounds (OOPC) on the immunogenicity of the LDL lipoprotein to autoantibody generation.

2. Methods

The EUROLIVE study was a multicentre, randomized, crossover intervention trial designed to assess the effect of sustained doses of olive oil, as a function of the phenolic compound (PC) content, on lipids and lipid oxidative damage. Participants were 200 healthy men, aged 20 to 60. Exclusion criteria were: the intake of antioxidant supplements or drugs with antioxidant properties, hyperlipidemia, obesity, diabetes, hypertension, intestinal disease, or any other condition that could impair adherence. Local institutional ethics committees approved the protocol and all participants provided written informed consent. The details of the EUROLIVE protocol have been described elsewhere.[2]

Participants were randomized to 3 consecutive interventions periods of raw olive oil administration (25 mL/day) with low (LPC, 2.7 mg/kg), medium (MPC, 164 mg/kg), and high (HPC, 366 mg/kg) polyphenol content. Each 3-week intervention was preceded by a 2-week wash-out period in which olive oil and olives were avoided. Participants were asked to avoid a high intake of antioxidant-containing food throughout the study period. The olive oils provided to participants had a similar composition of fatty acids, vitamin E, triterpenes, and sitosterol, differing only in their OOPC. Of the 200 participants, 182 (91%) completed the study protocol.

At the beginning of the study, and before and after each intervention period, blood samples were obtained at fasting state and 24-h urine was collected to gather the following data: 1) Glucose, total- and HDL-cholesterol and triglycerides measured by enzymatic methods, and LDL-cholesterol calculated by the Friedewald formula; 2) OxLDL by ELISA; F2-isoprostanes, by high-performance liquid chromatography (HPLC) and stable isotope-dilution and mass spectrometry; plasma C18 hydroxy-fatty acids, by gas chromatography coupled to mass spectrometry (GS–MS) and adjusted for LDL concentration; LDL uninduced conjugated dienes normalized to total cholesterol, by spectrophotometry and OLAB measured by ELISA using copper-oxLDL coated onto microtitter strips as antigen, and specific peroxidase- conjugated anti-human IgG antibodies to detect the presence of bound antibodies (Biomedica, Vienna, Austria); 3) Reduced and oxidized glutathione content of red cells determined by HPLC; and 4) Tyrosol and hydroxytyrosol, measured in 24-h urine by GS–MS. Finally, in a subsample of 36 participants, OOPC in LDL and metabolites were measured by HPLC-MS/MS, as previously described.[7]

Variables were log-transformed to achieve normality when appropriate. The relationship between tertiles of OLAB with lipids, lipid oxidative damage biomarkers, and baseline characteristics was assessed by one-way analysis of variance (ANOVA) test with polynomial contrast. Variables with a p-value ≤ 0.05 were then modeled to a linear regression analysis. The relationship between OLAB and the total content of OOPC in LDL was assessed by Pearson's correlation. The effect of the olive oil interventions on OLAB concentrations, adjusted by oxLDL, was assessed using a General Linear Mixed Model for repeated measurements adjusted for possible confounder variables. We performed all analyses on an intention-to-treat basis, using SPSS software, release 12.0 (SPSS Inc, Chicago, IL, USA).

3. Results

Baseline characteristics by OLAB tertiles are shown in Table 1. OLAB was inversely related with age, total- and LDL-cholesterol, and oxidized glutathione.

A strong inverse relationship was observed between OLAB and circulating oxLDL ($p < 0.001$) (Table 1). Changes in OLAB after each olive oil intervention were examined according to oxLDL tertiles. A significant interaction between the type of olive oil and oxLDL was observed, OLAB concentrations increasing with the phenolic content of the olive oil at high oxLDL concentrations ($p = 0.020$ for interaction) (Table 2). Participant compliance was good as reflected in the increase in urinary tyrosol and hydroxytyrosol concentrations ($p < 0.001$ for linear trend among interventions). Plasma OLAB concentrations correlated with the amount of OOPC bound to LDL-cholesterol, taking into consideration the values before and after LPC and HPC olive oils ($R = 0.209$; $p = 0.014$). Multiple linear regression analysis showed that the inverse relationship between oxLDL and OLAB was independent of other possible confounders (center, LDL-cholesterol and oxidized glutathione). Concentrations

Table 1
Baseline characteristics of the study participants by tertiles of OLAB.

Variable	OLAB 1st <306 U/L $n = 62$	OLAB 2nd [306–1099] U/L $n = 62$	OLAB 3rd >1099 U/L $n = 62$	P-value for linear trend
Age, years	37 (12)	33 (11)	30 (9)	0.020
Educational Level 1, (%) Tertiary education	21 (12.5%)	12 (7.3%)	15 (9.1%)	0.260
Educational level 2, (%) Primary and Secondary studies	37 (22.4%)	42 (25.5%)	38 (23%)	
Physical activity Kcal/day expended	320 (270)	262 (172)	303 (254)	0.494
Waist/Hip ratio	0.87 (0.06)	0.87 (0.05)	0.56 (0.06)	0.192
BMI, Kg/m2	24.3 (2.9)	23.8 (2.8)	23.5 (2.7)	0.108
SBP, mm Hg	124 (11)	123 (13)	125 (13)	0.428
DBP, mm Hg	77 (8)	76 (8)	77 (8)	0.697
Glucose mg/dL	87 (10)	83 (8)	87 (11)	0.181
Total cholesterol, mg/dL	194 (43)	182 (39)	173 (37)	0.025
HDL-cholesterol, mg/dL	49 (12)	45 (10)	47.5 (11)	0.979
LDL-cholesterol, mg/dL	125 (39)	119 (37)	107 (31)	0.046
Triglycerides, mg/dL	79.5 (62–114)	88 (64–115)	78 (58–109)	0.145
Oxidized LDL, U/L	55.7 (24.9)	49.6 (23.2)	43.5 (19.3)	0.001
Hydroxy-fatty acid, μmol/L	1.4 (0.6)	1.3 (0.4)	1.28 (0.40)	0.094
F2-Isoprostanes ng/L	30.29 (7.9)	29.1(5.3)	28.4 (6.6)	0.700
Uninduced conjugated dienes μmol/mmol of total cholesterol	2.41 (1.3)	2.70 (1.04)	3.01 (1.23)	0.058
Oxidized glutathione μmol/L	1.30 (0.14)	1.22 (0.10)	1.23 (0.09)	0.050

Values are expressed as MEAN (SD), MEDIAN (25th-75th percentile) or %; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoproteins; LDL, low density lipoproteins.

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Table 2
Changes (mU/L) in OLAB concentrations by baseline tertiles of oxidized LDL after low (LPC), medium (MPC) and high (HPC) phenolic content olive oil interventions.

Tertiles of oxidized LDL	LPC	MPC	HPC	*p*-value for linear trend	*p*-value for interaction between type of olive oil and tertiles of oxLDL
T1 <33.8 U/L	−47 (59)	111 (46)	−61 (69)	0.571	0.020
T2 [33.8 U/L-54.1 U/L]	−58 (76)	55 (50)	−7 (70)	0.088	
T3 >54.1 U/L	70 (91)	80 (79)	177 (95)	0.068	

Values are expressed as mean (SEM) and adjusted by centre, age, LDL baseline and OLAb baseline; *p*-value of the interaction between tertiles of oxLDL and olive oil interventions, adjusted for each tertile by centre, age, LDL baseline and OLAB at baseline.

of OLAB were, therefore, adjusted by oxLDL. OLABs increased from baseline after each intervention reaching significance after the HPC intervention. Globally, OLAB concentrations increased directly, in a dose-dependent manner, with the polyphenol content of the olive oil administered ($p = 0.023$ for linear trend) (Fig. 1).

4. Discussion

A regular daily dose of olive oil, similar to the daily consumption recommended by the U.S. Food and Drug Administration, increased OLAB plasma concentrations in a dose-dependent manner as the OOPC increased.

OLAB are generated as a response to oxLDL. Thus, as a primary hypothesis, OLAB concentrations would be expected to increase with increasing LDL oxidation. However, we observed an inverse relationship between circulating oxLDL concentrations and OLAB. This fact has been previously observed in healthy volunteers[6] using the same methods and antibodies as in our study. Also in this sense, an inverse relationship between OLAB and the carotid intima-media thickness (IMT), a recognized marker for CHD, has been observed.[8] In contrast, a direct relationship between OLAB concentrations and oxidative status situations, such as myocardial infarction and carotid atherosclerosis progression, has also been described.[9] Several factors could explain the discrepancies observed in the relationship between oxLDL and OLAB concentrations, including type of population, lack of standardized assays, and differences in methods and antigens used for OLAB determination.[10] The fact that an increase in oxLDL is associated with a decrease in OLAB in this study and others could be due to the presence of soluble oxLDL-OLAB immune complexes that may be undetectable with our OLAB methodology.[11] The "clearance hypothesis" postulates a physiological function of OLAB in removing oxLDL particles from the systemic circulation by means of these OLAB-OxLDL immune complexes[11] resulting in low concentrations of free detectable OLAB. This protective role of OLAB on atherosclerosis generation, although not definitively established, is supported by data from human studies.[12]

In our study there was an inverse relationship between OLAB and age. Other reports have also shown a trend of lower OLAB in the elderly compared to young population.[13] In agreement with our previous studies,[14] total and LDL-cholesterol were inversely related to OLAB in this study. From our results, high OLAB levels are concomitant with a low oxidative status. In this sense, oxidized glutathione concentrations were the lowest at the higher tertile of OLAB.

Also here, an increase in OLAB concentrations, associated with the OOPC, was observed. In our experience, low concentrations of OOPC (3.3 mg/day during 3 weeks) administered to healthy volunteers did not promote OLAB changes.[15] However, an increasing trend in OLAB concentrations appeared when 7.1 mg/day of OOPC was administered to stable CHD patients during 3 weeks.[16] When, in this study, a higher OOPC amount was provided (8.1 mg/d, 3 weeks) to healthy volunteers, a significant increase of OLAB was observed after the intervention. The greatest impact of OOPC on increasing OLAB values was observed in subjects with high concentrations of oxLDL.

The concentration of OLABs in the circulation depends on the concentration and degree of LDL oxidation as well as the subject's individual immune response. From our results, on one hand, an immune response stimulation linked with the OOPC could increase OLAB levels. On the other hand, the antioxidant effect of OOPC could reduce the LDL oxidation with a concomitant decrease in the "clearance" of free OLAB, via OLAB-oxLDL complexes. Both mechanisms could account for the increase in free OLAB detected. Phenolic compounds from food have been shown to stimulate both innate and adaptive responses[17] via an increase in serum antibodies (IgM and IgG) and in the number of antibody producing cells. In our study, after adjusting for oxLDL concentrations, we observed an increase in IgG OLAB that was dose-dependent on the OOPC. This fact, together with the relationship observed between OLAB and the OOPC in LDL, suggests that an immune-stimulating effect of OOPC on OLAB generation could occur in vivo.

The large sample size of our study allowed us to assess the role of olive oil polyphenols in promoting OLAB concentrations in plasma. Results were modest, as expected from real-life doses of natural olive oils and the healthy status of the volunteers. A limitation of our study was the inability to measure OxLDL-OLAB immune complexes, which could have contributed to a stronger interpretation of the results.

In summary, in our European populations we observed an inverse relationship between oxLDL concentrations and OLAB. OLAB concentrations were directly related with the OOPC content in LDL. OLAB concentrations increased in a dose-dependent manner with the phenolic content of the olive oil administered. High lipid

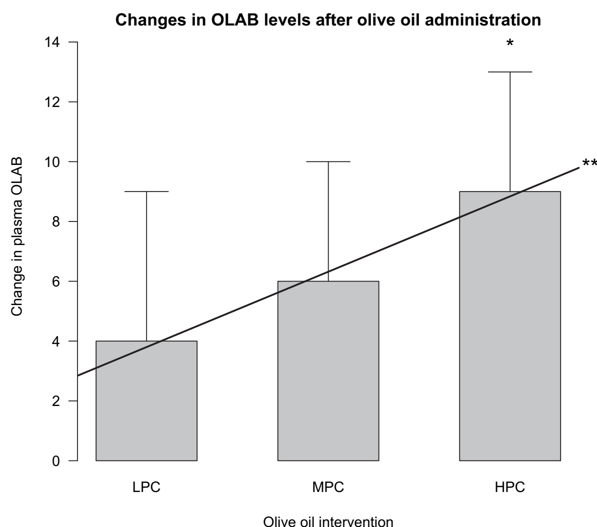

Fig. 1. Title: "Changes in OLAB levels after olive oil administration" Changes in OLAB concentrations (mIU/IU of oxLDL), after low (LPC), medium (MPC), and high (HPC) phenolic content olive oils. Values are expressed as mean ± SEM and *p*-value for linear trend from baseline. The model was adjusted by center, age, and LDL. *$p < 0.05$ change in OLAB after the HPC intervention compared to its basal. **$p = 0.023$ for linear trend.

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oxidative damage was associated with the greatest increase of OLAB values by olive oil polyphenols. On the basis of our results two mechanisms could be implied in high OLAB levels linked to high OOPC: 1) an immune response stimulation by OOPC, and 2) a lack of clearance of free OLABS due to a reduction in oxLDL levels by OOPC. A direct protective role of OLAB on atherosclerosis generation has been previously established and our results provide further support to recommend the use of polyphenol-rich olive oil as a source of fat, particularly for individuals presenting a high oxidative status.

Conflict of Interest

None declared.

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Statement of authorship: O.C carried out data analyses and wrote the manuscript; M.F carried out the collection of data, data analysis and supervision of the manuscript; MC.L-S carried out data analysis and contributed to the design of the experiment; HE.P participated in the design of the experiment and data collection; K.N participated in the design of the experiment and data collection; H.S helped to draft the manuscript and provisioned significant advise; JT.S participated in the design and data collection; K.T-C carried out samples analyses; H.F-Z collaborated in data collection; R.T participated in the design of the experiment and data collection as well as samples analyses; H.B. participated in the design and data collection; A-V.G collaborated with data collection; G.S carried out samples analyses; M.T reviewed the manuscript and MI.C conceived the study and elaborated its design and coordination and supervised the manuscript. All authors read and approved the final manuscript.

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